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Purification and Characterization of a Cytosol Protease from Dormant Cysts of the Brine Shrimp *Artemia**

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Alden H. Warner and Vijayalakshmi Shridhar

From the Department of Biology, University of Windsor, Windsor, Ontario, Canada N9B 3P4

A thiol protease has been isolated and purified from the postribosomal fraction of encysted embryos of the brine shrimp *Artemia* using a six-step procedure. The purified enzyme has a molecular weight of 55,000 ± 4,200 and is composed of subunits of M_r 31,500 ± 559 and 25,867 ± 1,087. Isoelectric focusing revealed two discrete bands, one at pH 4.6 and the other at pH 5.1. The protease appears to be a member of the thiol group of proteases based on its inhibition by leupeptin, antipain, chymostatin, Ep-475, and several other thiol protease inhibitors. The enzyme was stimulated by heavy metal chelators and thiol reagents. At pH 3.5–4.0 the thiol protease hydrolyzed a wide range of proteins including bovine serum albumin, hemoglobin, *Artemia* embryo soluble proteins, *Artemia* lipovitelline, and protamine, whereas at pH 6.0–6.5 the enzyme showed a high degree of specificity for *Artemia* elongation factor 2 and lipovitelline α1. The total amount of protease activity in crude homogenates of *Artemia* embryos decreased by about 50% during the first 24 h of development, while the amount of free, active enzyme decreased proportionally for 9 h of development then remained constant during the next 26–27 h of development. These changes in protease activity appear to reflect changing levels of an endogenous protease inhibitor during development.

Proteases have been implicated in a variety of metabolic reactions including activation reactions, regulatory reactions, and degradative reactions. (For reviews on these subjects see Refs. 1–6). Most of our knowledge concerning proteases has come from biochemical studies using mammalian tissues and a variety of microorganisms, whereas little, by comparison, is known about proteases from invertebrates despite the large number of species in this group of animals (7). Moreover, very few invertebrate oocyte or embryo proteases have been isolated, purified, and characterized except for those associated with the fertilization process (8). Despite the paucity of data on invertebrate embryo proteases, it has been presumed that the mobilization of yolk proteins during development is mediated by proteases within these embryos (9, 10).

The abundance of *Artemia* dormant cysts and the ease with which these embryos can be cultured into larval forms make this organism ideally suited for studies on the role of proteases during invertebrate development.

Previous reports have shown that dormant *Artemia* embryos contain at least three hydrolases/proteases active on proteins as substrates. One protease is found predominantly in the cytosol (11, 12), one has been reported to be associated with lipovitelline (13), and the third is reputed to be lysosomal (14). After hatching of nauplii at least four additional proteases become detectable in association with enhanced metabolic activity (15, 16).

In our efforts to learn more about the role of proteases in developing embryos, we have purified and characterized the cytosol protease from *Artemia* encysted embryos. The results presented here show that the cytosol enzyme from *Artemia* cysts is a thiol protease with a range of substrate specificities that is highly pH dependent. Our findings also suggest that the cytosol protease in *Artemia* plays a central role in yolk platelet utilization and protein synthesis regulation during development.

EXPERIMENTAL PROCEDURES AND RESULTS[1,2]

Characterization of the Artemia Cyst Cytosol Protease—The activity of the purified cytosol protease has been studied under a variety of conditions and with several substrates, both natural and synthetic. When the effect of pH on the protease activity was tested using two proteins and one peptide as substrate, the enzyme showed a sharp pH optimum between 3.6 and 3.8 with bovine serum albumin whereas with BANA or total soluble acidic proteins from *Artemia* the protease showed a broad pH optimum between 3 and 4.5. In all cases the enzyme showed no activity above pH 6.5.

In experiments to measure the stability of the enzyme at various pH values we found the *Artemia* protease to be most stable at pH 6.0 (in potassium phosphate buffer) and inactivated rapidly when incubated at pH 8.0 or above at 40 °C. When incubated at pH 4–5 in the absence of other proteins (at 40 °C), it rapidly underwent autolysis. The protease was found to be stable at pH 6 to freezing or storage for a few

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[1] Portions of this paper (including "Experimental Procedures," part of "Results," Fig. 1, and Tables I–III are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3060, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin; BANA, N^{α}-benzoyl-DL-arginine-β-naphthylamide; BAPNA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide; *N*-Cbz, *N*-carbobenzoxy; NA, naphthylamide; TAME, *p*-toluene sulfonyl-L-arginine methyl ester; TPCK, N^{α}-*p*-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; STI, soybean trypsin inhibitor; E-64, *N*-[*N*-(1,3-*trans*-carboxyoxiran-2-carboyl)-L-leucyl]-agmatine; TEMED, *N,N,N',N'*-tetramethylethylenediamine; EF, elongation factor; LV, lipovitelline.

days at 0–5 °C at concentrations greater than 0.5 mg/ml.

The temperature optimum for the hydrolysis of dimethyl bovine serum albumin at pH 4 was found to be 45 °C.

The molecular weight of the native enzyme was determined to be 55,000 ± 4,200 by gel filtration on Sephadex G-150 (superfine), while electrophoresis of the enzyme in SDS-polyacrylamide gels showed the enzyme to be composed of two subunits of M_r 31,500 ± 559 and 25,867 ± 1,087. (See Fig. 1*D* in Miniprint Section). The subunits were not active (enzymatically) when assayed in the standard reaction mixture with bovine serum albumin as substrate.

When the most purified preparations of the *Artemia* cyst soluble protease were analyzed in isoelectric focusing gels, two discrete bands were detected, one at pH 4.6 and the other at pH 5.1. These results are shown in Fig. 2. When a gel containing the focused protease was attached to the top of a slab gel containing 10% polyacryalmide and 0.1% SDS and electrophoresed in the vertical direction, two bands corresponding to the 31,500 and 25,900 subunits were observed from each of the focused bands (data not shown). In some experiments as many as seven additional bands were detected on the isoelectric focusing gel in the pH range of 4.7–5.9, but these bands did not contain subunits corresponding to either of the protease subunits found in the native enzyme.

The activity of the purified cyst cytosol protease has been studied under a variety of conditions using several substrates, both natural and synthetic. The results of experiments using six proteins and five peptides are shown in Table IV. Of the 11 potential substrates tested, protamine sulfate was found to be the most suitable protein while Cbz-Arg-Arg-NA was the most active peptide. The trypsin substrates TAME and BAPNA were found to be totally inactive with the *Artemia* cytosol protease, whereas BANA, an often used substrate for cathepsins B, L, and H was found to be slightly active. The K_m values for the hydrolysis of BANA, Cbz-Arg-Arg-NA, and bovine serum albumin were found to be 0.13, 0.24, and 0.012 mM, respectively, while the turnover rate constants, K_{cat}, were determined to be 2.09, 15.9, and 273, respectively, for the above substrates. The specificity factor, K_{cat}/K_m, showed that the *Artemia* acid protease hydrolyzes Cbz-Arg-Arg-NA more efficiently than BANA, but that it prefers protein as substrate.

The sensitivity of the protease to various substrates was found to vary widely with pH. In the pH range of 3–5 the *Artemia* cytosol protease catalyzed the hydrolysis of a wide range of proteins (Table IV) to small peptide fragments as

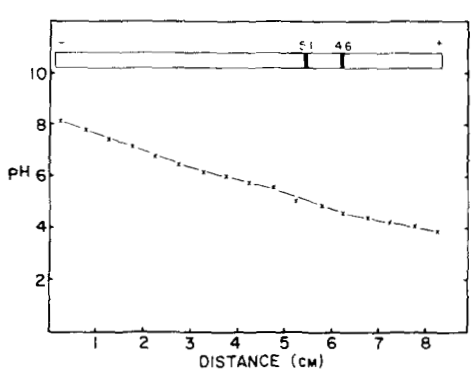

FIG. 2. **Determination of pI values on isoelectric focussing gel.** The migration position of the purified *Artemia* cyst cytosol protease is shown diagrammatically after electrophoresis for 18 h in a column of 5.2% polyacrylamide (4 × 85 mm) containing 2% of pH 3–10 Ampholine. The pH gradient was determined by measuring the pH of 0.5-ml H_2O extracts of 5-mm gel sections. The bands were visualized after staining with 0.1% Coomassie Brilliant Blue in 50% methanol and 7% acetic acid.

TABLE IV

Activity of Artemia cyst cytosol protease on various peptides and proteins

Substrate	pH	[*S*]	Product released
			nmol min^{-1} *mg*$^{-1}$
BANA	5.0	0.34 mM	17.3
N-Cbz-Arg-Arg-NA	5.0	0.34 mM	67.0
BAPNA	5.0	1.0 mM	Nil
TAME	3.9	0.33 mM	Nil
N-Cbz-Glu-tyrosine	5.0	15 mM	Nil
Bovine serum albumin	4.0	4 mg/ml	2516
Dimethyl bovine serum albumin	4.0	4 mg/ml	2830
Hemoglobin	4.0	4 mg/ml	3650
Artemia lipovitellins	4.0	4 mg/ml	3594
Protamine sulfate	4.0	4 mg/ml	6500
Artemia elongation factor 2	6.0	0.45 mg/ml	[a]

[a] Observed by SDS-polyacrylamide gel electrophoresis (see Fig. 3).

observed by SDS-polyacrylamide gel electrophoresis (data not shown). However, between pH 6.0–6.6 the protease displayed a high degree of specificity for selected *Artemia* proteins. In this pH range the purified cytosol protease was observed to hydrolyze the protein synthesis elongation factor, EF-2 (M_r 94,000), rapidly into fragments of M_r 54,000 and 40,000 (inactivating the factor in protein synthesis) whether tested in the presence or absence of other soluble acidic proteins from *Artemia* embryos. Subsequently these polypeptide fragments were hydrolyzed slowly into polypeptide fragments of M_r 45,000 and 38,000. These results are shown in Fig. 3. Other major soluble acidic cytosol proteins of *Artemia* were found to be resistant to hydrolysis by the cytosol protease at pH 6.0–6.5. When [^{3}H]adenosine diphosphate ribosyl-EF-2 was taken as substrate and SDS-polyacrylamide gel electrophoresis used to separate the hydrolytic products produced at pH 6.5, we observed the appearance of ^{3}H only with fragments of M_r 40,000 and 38,000. When lipovitelline isolated from *Artemia* cyst yolk platelets was tested as substrate at pH 6.0–6.5 and the reaction mixtures analyzed by SDS-polyacrylamide gel electrophoresis, we observed that LV-α1 (M_r 185,000), the largest of the two major lipovitellins, was hydrolyzed sequentially to LV-α10 (M_r 120,000). The other major lipovitelline in the yolk platelets of *Artemia* cysts (LV-ϵ, M_r 61,000) was found to be resistant to hydrolysis by the cytosol protease at pH 6.0–6.5, while two minor lipovitellins (LV-δ1, M_r 83,500; LV-δ2, M_r 73,000) were also hydrolyzed. These findings are shown in Fig. 4. All lipovitellins were hydrolyzed to small peptides when incubated at pH 4–5. Of interest was the fact that the pattern of lipovitelline hydrolysis by the cyst cytosol protease *in vitro* at pH 6.0–6.5 was similar to that observed *in vivo* during development of *Artemia* to the nauplius stage (13, 32).

In an effort to characterize the *Artemia* cytosol protease further, the enzyme was treated with or assayed in the presence of a wide variety of reagents. The results of these experiments are shown in Table V. The enzyme was found to be insensitive to serine protease inhibitors such as ovomucoid, soybean trypsin inhibitor, and TPCK but sensitive to thiol protease inhibitors such as leupeptin, antipain, chymostatin, Ep-475, and iodoacetate.

The thiol-blocking reagents pCMB and NEM had little effect on the activity of the protease at 1 and 10 mM, respectively, while the thiol reagents DTT and mercaptoethanol showed a slight stimulatory effect as did the heavy metal chelators EDTA and 1,10-phenanthroline. The protease was

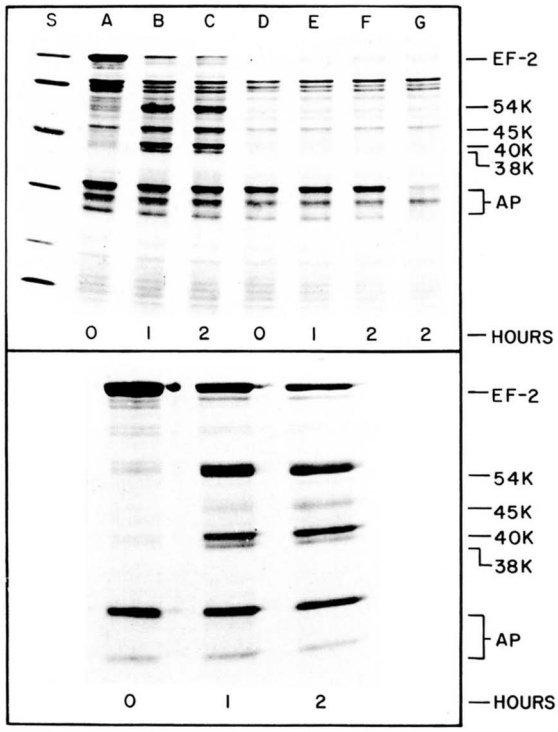

FIG. 3. **Selective hydrolysis of *Artemia* EF-2 by the acid protease at pH 6.0–6.5.** *Top panel*, incubation of *Artemia* soluble acidic proteins (60 μg) with the acid protease (4.5 μg) and added EF-2 (8 μg) (*A–C*) or without added EF-2 (*D–G*) at 30 °C and pH 6.5. *Column G* did not contain added acid protease (*AP*). *Column S* represents standard molecular weight proteins. From *top* to *bottom* they are: phosphorylase (M_r 94,000), bovine serum albumin (M_r 67,000, ovalbumin (M_r 43,000) carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400). *Bottom panel*, incubation of EF-2 (8 μg) with acid protease (4.5 μg) only at 30 °C and pH 6.0.

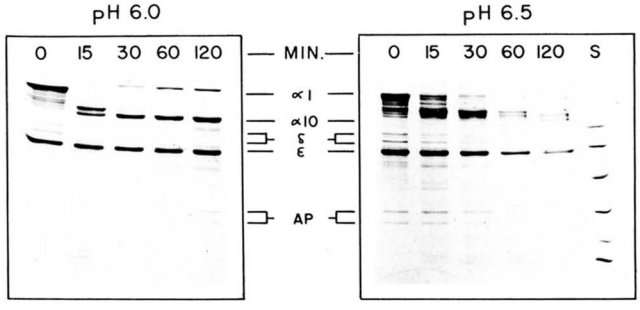

FIG. 4. **Pattern of *Artemia* lipovitelline hydrolysis by the acid protease at pH 6.0–6.5.** Lipovitelline from *Artemia* (12 μg) was incubated at pH 6.0 and pH 6.5 with *Artemia* acid protease (1.9 μg) at 30 °C. *Column S* represents standard molecular weight protein markers as described in the legend to Fig. 3. *AP*, acid protease.

found to be insensitive to the carboxyl protease inhibitors PMSF, pepstatin, and TPCK. Only iodoacetate among the nonpeptide class of thiol inhibitors was effective in blocking the protease activity. These results together with previous data showing Cu^{2+} and Hg^{2+} to be potent inhibitors of the enzyme (12) suggest that the *Artemia* cyst cytosol protease is a thiol protese. Partially purified preparations of two protease inhibitors from *Artemia* cyst cytosol were also found to be inhibitory at low inhibitor concentrations. Details of these findings will be presented in a future report.

Artemia embryos are unable to tolerate high concentrations of potassium salts in their aquatic environment, but they are

TABLE V

Effect of various protease inhibitors and other reagents on Artemia acid protease

Treatment	Concentration	% of control
Ovomucoid	1.0 mg/ml	97
Soybean trypsin inhibitor	1.0 mg/ml	98
Leupeptin	4.0×10^{-4} mM	50
Antipain	7.1×10^{-4} mM	50
Ep-475	3.5×10^{-4} mM	50
Chymostatin	0.3 μg/ml	50
PMSF	1.3×10^{-1} mM	100
Pepstatin	1.5×10^{-2} mM	100
TPCK	5.0×10^{-2} mM	80
TLCK	5.0×10^{-2} mM	3
Artemia protease inhibitor A[a]	27 μg/ml	50
Artemia protease inhibitor B[a]	47 μg/ml	50
Iodoacetate	1.0 mM	13
pCMB	1.0 mM	88
NEM	10.0 mM	96
EDTA	1.0 mM	114
1,10-Phenanthroline	1.0 mM	157
DTT	1.0 mM	117
β-Mercaptoethanol	1.0 mM	109
β-Mercaptoethanol	5.0 mM	131
SDS	0.01%	100
SDS	0.10%	5
Urea[b]	7 M	0

[a] Assayed at pH 5.0 after purification through DEAE-cellulose, CM-cellulose, G-75 Sephadex.

[b] Enzyme (1 mg/ml) treated with 7 M urea (0 °C) for 1 h followed by dialysis overnight to remove urea.

resistant to high concentrations of sodium salts. Therefore, we decided to test the effect of two potassium and one sodium salt on the activity of the *Artemia* cyst protease. Our results indicate that both NaCl and KCl inhibit the protease activity with similar kinetics while KI appears to be somewhat more inhibitory at 50 and 100 mM than either KCl or NaCl at pH 4.0. However, in all cases 15–18% of the protease activity was found to be resistant to salt concentrations ranging to 0.75 M. Similar experiments carried out at pH 5.0 showed the salt effects to be minimal at the higher pH.

Developmental Study of Artemia Cytosol Protease—The results of a developmental study of the cytosol protease level in crude extracts (postribosomal fraction) and inhibitor-free preparations (after chromatography on DEAE-cellulose) are shown in Fig. 5. These data show that the acid protease activity in the postribosomal fraction (free enzyme) declined by about 50% during the first 9 h of incubation following resumption of development, then remained relatively constant through the next 26–27 h of development (Fig. 5*B*). In contrast, the total amount of acid protease in developing embryos was found to decrease continuously during the first 24 h of development. The profile of alkaline protease activity in the postribosomal fraction was also measured. This is also shown in Fig. 5*B* for comparison. Of particular interest is the finding that 35–45% of the total protease activity (acid) is complexed in extracts of pre-emerged embryos, while the distribution of free and bound enzyme changes dramatically concomitant with emergence of the prenauplius larva (Fig. 5*C*). These findings suggest that the expression of the cytosol protease is highly regulated in pre-emerged larva.

DISCUSSION

Protein breakdown and turnover in *Artemia* embryos probably involves multiple proteases and pathways as in other systems (4). Moreover, the activity of these proteases is probably controlled to a marked extent by the presence of intra-

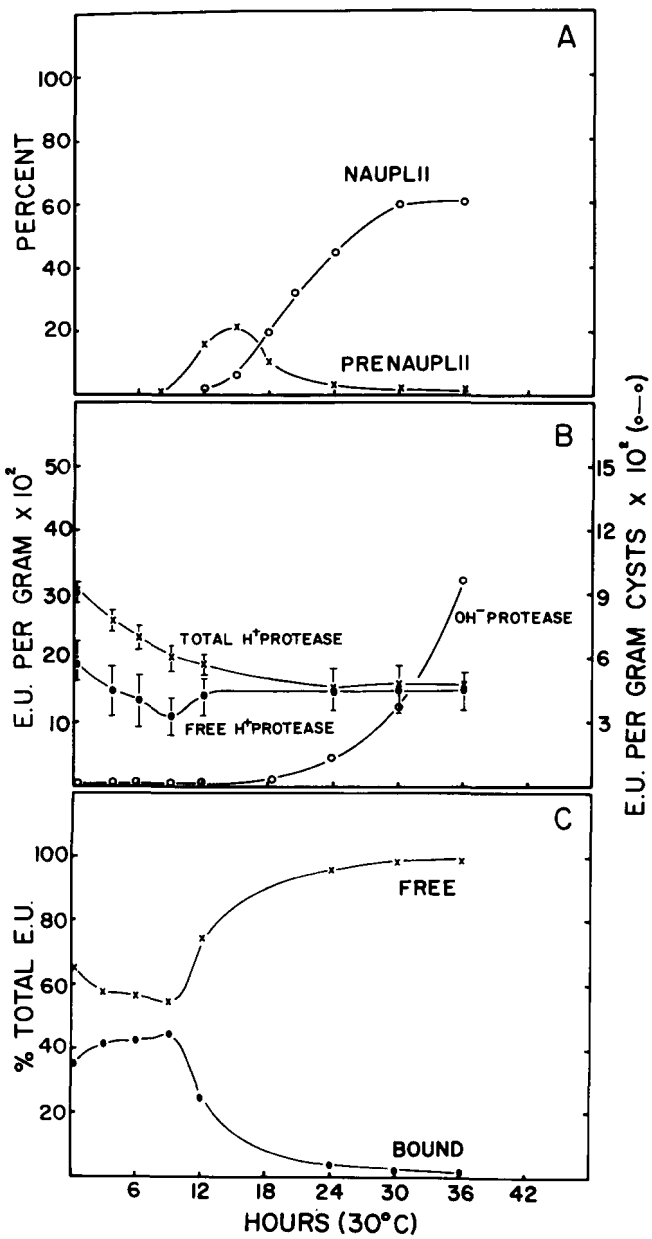

FIG. 5. **Protease activity in biochemical preparations from *Artemia* embryos at various stages of development.** *A*, developmental profile of prenauplii and nauplii used in these experiments. *B*, level of free acid protease (in postribosomal fraction after dialysis) and total acid protease (after DEAE-cellulose treatment to remove endogenous inhibitor) compared to alkaline protease activity at various stages of development. *C*, per cent enzyme in the "free" and "bound" form calculated from data in *B*. In *B* the enzyme units are expressed in terms of grams wet weight (fully hydrated) of starting cysts, and each point represents the average of four determinations.

cellular inhibitors and intracellular pH (11, 33). In dormant and prehatched embryos of the brine shrimp *Artemia* one protease has been found in amounts large enough to purify and study in detail. This protease, first described by Nagainis and Warner (11), has been purified to homogeneity and partially characterized. It belongs to the thiol group of proteases and it is active on BANA, N-Cbz-Arg-Arg-NA, and proteins as substrate; it is more active on protein substrates than on arginine-containing peptides (34). It has a pH optimum considered low for thiol proteases (3.5–4.0), but it possesses a high degree of specificity for certain proteins in the pH range of 6.0–6.5. Unlike cathepsin B of vertebrates, the *Artemia* thiol protease was found predominantly (61–81%) in the postribosomal fraction although some activity (14–19%) was found in the mitochondria-rich fraction.

The thiol protease described here has many properties in common with the thiol protease isolated and purified from the particulate fraction of *Artemia* cysts by Perona and Vallejo (14). The main differences in our results and those of Perona and Vallejo are in molecular weight, subunit composition, and sensitivity to KI. The particulate enzyme, which they reported to be lysosomal, has a molecular weight of 68,000 and is composed of a single subunit. The cytosol enzyme described in this report has a molecular weight of 55,000 and is composed of subunits of 31,500 and 25,867 units. The particulate enzyme is completely inhibited by 1 mM KI while the cytosol thiol protease reported here is unaffected by 1 mM KI. These observations suggest the presence of two distinct thiol proteases in *Artemia* cysts. However, it should be noted that Perona and Vallejo used *Artemia* cysts treated with NaOCl as their starting material for protease isolation. Workers in our laboratory have shown that this sterilization treatment causes artifacts in measurements of the thiol protease activity (see Table II in Miniprint Section) and other sulfhydryl-rich proteins such as EF-2 (31). Similar problems have also been encountered in DNA polymerase studies.[3] Thus, it is our view that our method of isolation and purification yields the *Artemia* cyst cytosol thiol protease in its native form.

One of the major developmental events occurring in *Artemia* following resumption of development is yolk platelet utilization. During the first 24 h of incubation of *Artemia* cysts at 30 °C there is about a 50% decrease in the number of yolk platelets in these embryos (from 773 to 361 × $10^7/10^5$ embryos) (35). Concomitant with the reduction in the number of yolk platelets is hydrolysis of the major lipovitelline in yolk (LV-α1, M_r 190,000) to polypeptides ranging in molecular weight to M_r 120,000 (LV-α10). Also, while Vallejo and Marco (32) reported that an endogenous alkaline protease in *Artemia* yolk platelets is responsible for the hydrolysis of LV-α1 (M_r 190,000) to LV-α10 (M_r 120,000), we have been unable to confirm these findings. Rather, our data suggest that the cyst cytosol thiol protease is active at the yolk platelet level in the sequential hydrolysis of LV-α1 to LV-α10 at pH 6.0–6.5. This conclusion is supported by resonance data from ^{31}P-NMR studies which show the presence of at least one acidic compartment in *Artemia* embryos (33).

In the study reported here we also demonstrated that the cytosol protease has a preference for *Artemia* EF-2 as substrate at pH 6.0–6.5. These findings suggest that the cyst thiol protease controls the rate of protein synthesis in embryos subjected to anoxia in which the intracellular pH (pH_i) decreases to about 6.8 within 90 min of the onset of anoxia. Since protein synthesis is switched off during anoxia[4] under conditions (pH 6.5) in which EF-2 is inactivated (*in vitro*) by the thiol protease, activation of the thiol protease *in vivo* may function in protein synthesis shutdown. In fact Bernaerts[5] in this laboratory observed a 10-fold increase in acid protease activity in the postribosomal fraction of hydrated *Artemia* gastrulae within 90 min after exposure of the embryos to anaerobic conditions which lowered the pH_i to 6.8. Since the increase in protease activity could not have been due to an increase in protein synthesis, it must have resulted from a pH-induced shift in equilibrium between the free and inhibited forms of the protease.

The importance of the intracellular protease inhibitor has

[3] A. G. McLennan, personal communication.
[4] A. H. Warner and V. Shridhar, unpublished observations.
[5] F. Bernaerts, unpublished data.

yet to be determined, but it seems certain that this inhibitor plays a vital role in the control of protease activity during development of *Artemia*.

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Supplemental Material To:
Purification and Characterization of a Cytosol Protease from Dormant Cysts of the Brine Shrimp Artemia.

A. H. Warner and V. Shridhar

EXPERIMENTAL PROCEDURES

Materials - Artemia dormant cysts, purchased from Longlife Aquarium Products Harrison, N.J. in 1970 and stored dessicated at -20°C, were used as starting material. About 50% of these cysts gave rise to swimming nauplii after 24 hours incubation in sea water at 30°C (17). The substrates tested in this study were as follows. BSA[2], hemoglobin, protamine sulfate, BANA, BAPNA, TAME, N-CBZ-arg-arg-NA and N-CBZ-glu-tyr were from Sigma. The protease inhibitors pepstatin and leupeptin were generous gifts from W. Troll (New York University) and the U.S. -Japan Cooperative Cancer Research Program, while E_p475, a synthetic analogue of E-64, was kindly supplied by K. Hanada (Taisho Pharmaceutical, Japan). All other known protease inhibitors including ovomucoid, STI, antipain, chymostatin, PMSF, TPCK, TLCK, iodoacetate, pCMB, and NEM were from Sigma. Sephadex G-25, G-150 and DEAE-Sephadex were from Pharmacia, while phosphocellulose P11 was from Whatman and hydroxyapatite was from Clarkson Chemical Co. Acrylamide, bisacrylamide, TEMED and SDS were from Bio-Rad. TNBS, fast garnet GBC salt and Brij were from Sigma. All other chemicals were reagent grade. A Sorvall RC2-B centrifuge was used for all procedures requiring low speed centrifugation (less than 40,000 x g) while all steps requiring high speed centrifugation (greater than 40,000 x g) were carried out using a 60Ti rotor in a Beckman ultracentrifuge (Model L5-65). All UV-and visible light measurements were carried out using a Beckman Spectrophotometer (Model 25) with a sipper cell attachement.

Artemia Embryo Proteins - Yolk platelets were isolated from dormant cysts of Artemia using the aqueous procedure of Warner et al. (18). The lipovitelline soluble in 1M NaCl buffered with 25mM potassium phosphate, pH 7.0, was obtained as described previously (19). Protein synthesis elongation factor 2 (EF-2) was purified from Artemia cysts as described by Yablonka-Reuveni et al. (20). The total soluble acidid proteins referred to in the Results refer to those Artemia cyst proteins which bind to a DEAE-cellulose column at pH 6.8 (μ = 0.04) and which elute with 0.5M KCL. This fraction is free of Artemia acid protease inhibitor (11).

Enzyme Assays - During the purification procedure and developmental study the protease activity in various column fractions and embryo extracts was determined by the liberation of amino groups according to the TNBS method of Fields (21) as modified by Nagainis and Warner (11). Unless otherwise stated, all reaction vessels contained the following in 0.20 ml final volume: 0.1M sodium acetate, pH 4.0, 1mM EDTA, 0.8 mg N,N-dimethyl BSA and enzyme. The methylated BSA was prepared according to Lin et al. (22). The reaction vessels were incubated at 40°C and at the desired times, 0.05ml aliquots were removed and added to 0.95 ml of a solution containing 0.053M Na_2B_4O7 and 0.053M NaOH. Color development was initiated by the addition of 0.025ml of 0.22M TNBS and the tubes were allowed to remain at room temperature for 15 minutes. Color development was stopped by the addition of 2.0 ml of 0.1M NaH_2PO_4 containing approximately 1.5 mM Na_2SO_3. Color intensity was detemined immediately at 420 nm. One unit of protease activity is defined as the liberation of 1 nmole of amino groups per min. compared to alanine as standard. With substrates containing napthylamides (BANA, BAPNA, and N-CBZ-arg-arg-NA) the method of Barrett was used (23). The hydrolysis of BSA, hemoglobin, protamine sulfate, yolk proteins and N-CBZ-glu-tyrosine was followed using the TNBS assay while the hydrolysis of EF-2 was monitored by SDS-polyacrylamide gel electrophoresis and TAME according to the procedure of Hummel (24).

Electrophoresis - The purity of Artemia acid protease and selected reaction products were analyzed on 10% polyacrylamide gel slabs (100 x 135 mm) containing 0.1% SDS according to the procedure of Laemmli (25). Isoelectric focussing of the acid protease was carried out in gel columns (4 x 100 mm) according to Blerkom (26). Protein was stained with Coomassie brilliant blue (R-250) in 7.5% acetic acid and 50% methanol.

Molecular Weight Measurements - The molecular weight of the native protease was determined by gel filtration on a column of Sephadex G-150 (1.5 x 85 cm, superfine) according to the procedure of Andrews (27). The molecular weight markers used were γ-globulin (M_r 160,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000) and chymotrypsinogen (M_r 25,000). The molecular weight of the polypeptide subunits of the Artemia protease was determined by SDS-polyacrylamide gel electrophoresis using various combinations of the following standard molecular weight markers: β-galactosidase (M_r 130,000) phosphorylase (M_r 94,000), bovine serum albumin (M_r 67,000), γ-globulin H-chain (M_r 50,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), γ-globulin L-chain (M_r 23,500) soybean trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,400).

Subcellular Fractionation - The preparation of various cell fractions was undertaken to determine the subcellular localization of the Artemia cyst (acid) protease. Three buffer systems were used which had been shown previously to be effective in preserving various organelles intact (20,28,29). The details of these buffer systems are described in the Results.

Protein Determination
Protein was measured by the method of Lowry et al. (30).

RESULTS

Distribution of Acid Protease Activity in Artemia Embryos
Proteases active in the pH range 3 to 5 are usually found in lysosomes of animal cells. However, lysosomes have not yet been clearly identified in Artemia embryos although one group of investigators claim that they exist (14). Using three buffer systems to homogenize and fractionate Artemia embryos, we found that at least 60% of the total acid protease activity is localized in the post-mitochondrial/lysosomal fraction. Moreover, with homogenizations buffers often used to isolate lysosomes, we observed over 80% of the total acid protease activity in the post-mitochondrial/lysosomal fraction. These findings are summarized in Table I and suggest that the cyst protease (acid) is localized mainly in the cytosol and not in lysosomes or yolk platelets. For this reason the post-ribosomal fraction (150,000 x g supernate or S-150) has been used routinely as the starting source of this enzyme.

Table I. Distribution of Acid Protease Activity Among Various Artemia Embryo Fractions.

Cell Fraction	Buffer STKMD[a]	Buffer STE[b]	Buffer PK[c]
750 x g pellet	19.8	2.8	d
15,000 x g pellet	19.0	14.5	14.6
15,000 x g supernate	61.2	82.7	d
150,000 x g pellet	d	d	4.0
150,000 x g supernate	d	d	81.4

a This buffer contains 0.25M sucrose, 0.03M Tris-HCl, pH 7.3 (22°C), 5mM KCl, 10mM $MgCl_2$ and 1mM DTT

b This buffer contains 0.25M sucrose, 0.03M Tris-HCl, pH 7.3 (22°C), and 0.001M EDTA

c This buffer contains 0.015M potassium phosphate, pH 7.0 (22°C) and 0.1M KCl

d not measured

Effect of Cyst Sterilization with NaOCl on the Cytosol Protease Activity
In the preparation of Artemia cysts for biochemical studies, NaOCl or antiformin (which contains (NaOCl) is often used to remove microorganisms and chemically sterilize the cysts (14,17,31). The results shown in Table II demonstrate that cysts sterilized with NaOCl have significantly reduced protease activity (acidic) in both the unfractionated cytosol and fraction bound to DEAE-cellulose which eluted with 0.35M KCl buffered with 25 mM potassium phosphate, pH 6.8. Extensive washing of the NaOCl-treated cysts with 0.1N HCl, 1M urea or 5-20 mM mercaptoethanol failed to eliminate the damaging effect of NaOCl on the cytosol protease activity. The reason for the reduced acid protease activity of extracts from cysts treated with NaOCl is not known since Artemia cysts show a high hatching efficiency after sterilization by this method (17).

Table II. Effect of NaOCl Sterilization of Artemia Cysts on the Cytosol Protease Activity.

Step	Control			NaOCl Treated Cysts[b]			% of Control
	Total A_{280}	S.A. (EU/A_{280})	Total Enzyme Units[a]	Total A_{280}	S.A. (EU/A_{280})	Total Enzyme Units[a]	
15,000 x g supernate	85.0	5.6	476	66.0	3.2	211	44
Sephadex G-25	12.4	28	347	6.1	10	61	18
DEAE-cellulose	4.0	196	784	2.0	94	188	24

a Based on one gram (wet weight) hydrated cysts
b Hydrated cysts were treated with 5% NaOCl for 30 min. prior to washing sequentially with 750 ml H_2O, 50 ml 2 mM mercaptoethanol and 500 ml H_2O.

Purification of the Artemia Cyst Cytosol Protease
A six-step purification procedure has been developed which yielded a protease over 95% pure and representing about 1.4% of the cytosol proteins. The details of each step are described below and the results summarized in Table III.

Table III. Summary of Purification Steps for Cyst Acid Protease[a]

Step	Total protein (mg)	Specific Activity (Eu/mg)	Total Enzyme Units	Yield (%)
S-150	2870	33.6	96,400	100
40-55% $(NH_4)_2SO_4$	1410	62.2	87,700	91
Phosphocellulose	480	136	65,300	68
DEAE-Sephadex	96	588	56,400	59
Hydroxyapatite	28	1420	39,800	47
Sephadex G-150	7	2427	17,000	18

a The values are based on 100 grams dry cysts as starting material from two complete experiments using 50 grams dry cysts each.
b The enzyme activity in these fractions was determined on aliquots filtered through a G-25 Sephadex column (2 x 15 cm) equilibrated with 0.025 M KCl containing 0.015 M potassium phosphate, pH 6.8 to remove small molecular weight components.

Step 1: Preparation of Post-Ribosomal Fraction (S-150)
Hydrated and washed dormant cysts, in 25 gram batches were ground using a motorized mortar and pestle with 10 ml of 50 mM Tris-HCl, pH 7.3 containing 5 mM KCl, 1mM DTT and 10 mM $MgCl_2$ (Buffer A) until a thick and smooth paste resulted (about 10 minutes). The paste was transferred to a beaker and 115 ml of Buffer A was added and the homogenate stirred for 15 minutes. The process was repeated until 110 grams of wet cysts (equivalent to 50 grams dry weight) had been processed. The homogenate was centrifuged at 10,000 x g for 45 minutes and the resulting supernatant fluid, minus the floating lipid layer, was centrifuged at 150,000 xg for 2.5 hours. The resulting supernatant fluid (S-150) was found to contain over 60% of the total cysts acid protease activity (see Table I).

Step 2: Preparation of the 40-55% Ammonium Sulfate Fraction
The S-150 fraction was adjusted to 30 $A_{260/ml}$ by the addition of buffer A then solid ammonium sulfate was added to a final concentration of 40%. The preparation was stirred for 30 minutes and the precipitate was removed by centrifugation. The soluble fraction was adjusted to contain 55% ammonium sulfate and after stirring for 30 minutes the resulting precipitate was collected by centrifugation and saved for the next step. The 40-55% ammonium sulfate precipitate contained 91% of the acid protease activity in the S-150 fraction and it was relatively free of low molecular weight protease inhibitors found previously (11).

Step 3: Protease Fractionation on Phosphocellulose
The 40-55% ammonim sulfate precipitate was dissolved in a small volume of 0.025 M KCl containing 0.015 M potassim phosphate, pH 6.8 and filtered through a column of Sephadex G-25 (3.8 x 50 cm) equilibrated with the same buffer. The protein rich effluent was applied to a column of phosphocellulose (2.5 x 40 cm) equilibrated with the same KCl-phosphate buffer and washed with buffer until enzyme eluted from the column. Under these conditions the protease activity eluted immediately after the major protein peak and it was relatively free of orange pigment (see Figure 1, panel A). This step was found to be crucial to the purification scheme, for without it, we have been unable to obtain pure protease using a variety of fractionation procedures.

Step. 4. Purification of the Artemia Cyst Cytosol Protease on DEAE-Sephadex
The active column fractions from the phosphocellulose step were pooled adjusted to pH 7.0 with NaOH, then applied to a column of DEAE-Sephadex (1.8 x 34 cm) equilibrated with the same KCl-phosphate buffer used for the phosphocellulose step, except at pH 7.0. Following application of the protease fraction, the column was washed with 150 ml of the starting buffer and the bound proteins were eluted with a 500 ml linear gradient of KCl (25 mM to 750 mM) buffered with 0.015 M potassium phosphate, pH 7.0. This step yielded about 87% of the protease activity applied to the column in a symmetrical peak eluting between 0.25 and 0.35 M KCl (see Figure 1, panel B).

Step 5. Fractionation of the Cytosol Protease on Hydroxyapatite
The protease fractions from DEAE-Sephadex were pooled and applied directly to a column of hydroxyapatite (1 x 24 cm) equilibrated with the KCl-phosphate buffer used in the previous step. The column was washed with 50 ml of the starting buffer and bound proteins were eluted with a linear gradient of potassium phosphate, pH 7.0 (from 15 to 500 mM). The protease fractions which eluted with the second UV-absorbing peak (see Figure 1, panel C) were pooled and concentrated to less than 2 ml by vacuum dialysis. This preparation contained 47% of the acid protease activity in the S-150 fraction while only 1% of the protein.

Step 6. Purification of the Artemia Protease by Gel Filtration
The concentrated protease from hydroxyapatite was applied to a column of Sephadex G-150 (1.5 x 86 cm, 10-40 mesh) equilibrated with 0.025 M KCl containing 0.015 M potassium phosphate, pH 7.0 and eluted with this buffer at a flow rate of 2.0 ml/hr. The protease activity eluted as a symmetrical peak with a K_{av} identical to the third UV-absorbing peak to elute from the column (see Figure 1, panel D). The column fractions containing the active protease were pooled, concentrated by vacuum dialysis and stored at -20°C.

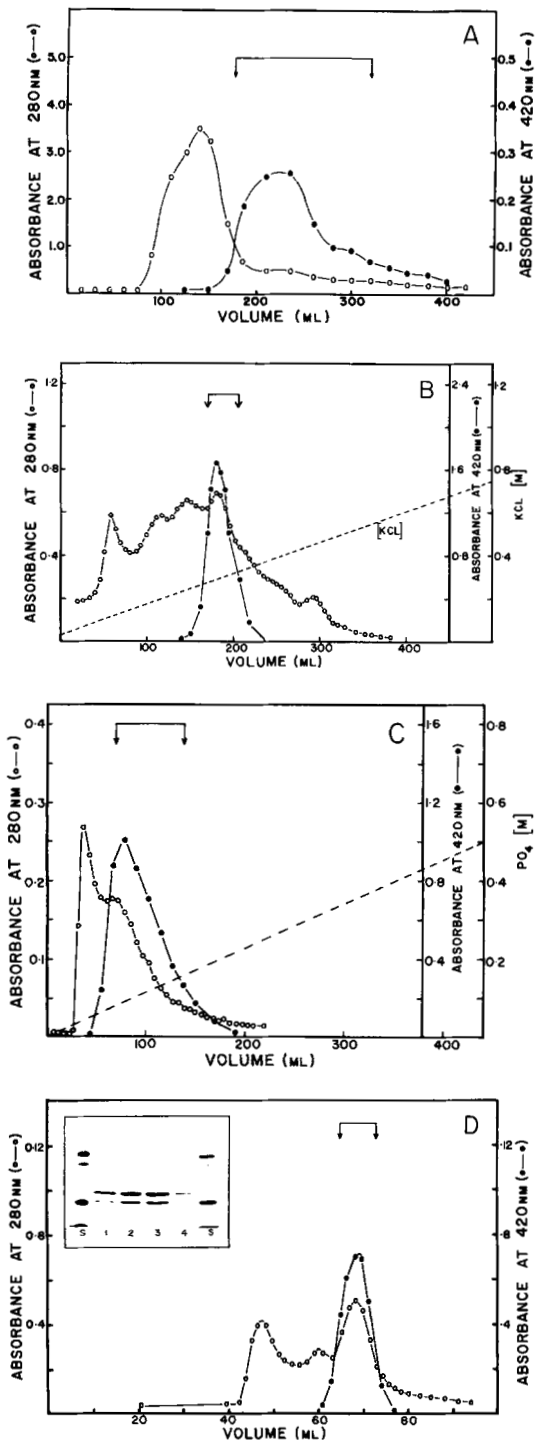

Fig. 1. Chromatographic steps in the purification of the Artemia cyst cytosol protease. A. Purification of the protease in the 40-55% ammonium sulfate precipitate from 50 grams dry cysts starting material on a column of phosphocellulose (2.5 x 40 cm) equilibrated with a solution containing 0.025 M KCl and 0.015 M potassium phosphate, pH 6.8. Following application of the protein in the equilibration buffer (about 50-60 ml), the column was washed with the equilibration buffer until the bulk of the protease activity eluted from the column. B. Purification of the protease on a column of DEAE-Sephadex (1.8 x 34 cm). The protease fraction from the previous step (panel A) was applied directly to the column and following washing of the column with 150 ml of the equilibration buffer at pH 7.0, the proteins were eluted using a linear gradient of KCl in 0.015 M potassim phosphate, pH 7.0 as shown in Panel B. C. Chromatography on hydroxyapatite. The active fractions from the previous step were applied to a column of hydroxyapatite (1 x 24 cm) equilibrated with the same KCl-phosphate buffer (pH 7.0) used in the previous step (see panel B). Following washing of the column with 50 ml of the starting buffer, the proteins were eluted with a linear gradient of potassium phosphate, pH 7.0, as shown in panel C. D. Purification of the protease by gel filtration. The protease fractions from the previous step were concentrated to less than 3 ml by vacuum dialysis then applied to a column of Sephadex G-150 (1.5 x 86 cm, 10-40 mesh) equilibrated with the same KCl-potassium phosphate buffer (pH 7.0) employed in the previous two equilibration steps. The protein was eluted from the column with the same buffer and selected column fractions were assayed for protein content and protease activity. The insert in panel D represents an SDS-polyacrylamide gel analysis of aliquots from the most active fractions in the third peak to elute from the column. S represents the following molecular weight markers from the top to the bottom of the gel: gamma globulin (HC; 50,000), ovalbumin (43,000), gamma globulin (LC; 23,500) and ribonuclease (13,700).

The arrows in each panel indicate the fractions pooled for subsequent purification and/or analysis. o---o, absorbance at 280 nm. •---•, protease activity measured at pH 4.0 using the standard (TNBS) enzyme assay.